

1. The first step is to identify the problem. This involves understanding the current situation and what needs to be changed.

UNITED STATES

Inventors: Neil Berinstein, James Tartaglia, Philippe Moingeon
and Brian Barber

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5 no. 60/160,879, filed on October 22, 1999 and United States application
serial no. 60/223,325 filed on August 7, 2000, both of which are incorporated
by reference herein.

FIELD OF THE INVENTION

10 enhancing immune responses to tumor antigens.

BACKGROUND OF THE INVENTION

of cancer immunotherapy rely upon the identification of tumor associated antigens ("TAA") which can be recognized by the immune system. Specifically, target antigens eliciting T cell-mediated responses are of critical interest. This comes from evidence that cytotoxic T lymphocytes (CTLs) can induce tumor regression both in animal models (Kast W. et al (1989) *Cell* 59:6035; Greendberg P. (1991) *Adv. Immunol.* 49:281) and in humans (Boon T. et al. (1994) *Annu. Rev. Immunol.* 12:337). To date, many tumor associated antigens have been identified. These include the antigens MAGE, BAGE, GAGE, RAGE, gp100, MART-1/Melan-A, tyrosinase, carcinoembryonic antigen (CEA) as well as many others (Horig and Kaufman (1999) *Clinical Immunology* 92:211-223). Some of these tumor associated antigens are discussed below.

The first human tumor associated antigen characterized was identified from a melanoma. This antigen (originally designated MAGE 1)

Human carcinoembryonic antigen (CEA) is a 180 kD glycoprotein expressed on the majority of colon, rectal, stomach and pancreatic tumors (Muaro et al. (1985) *Cancer Res.* 45:5769), some 50% of breast carcinomas (Steward et al. (1974) *Cancer* 33:1246) and 70% of lung carcinomas (Vincent, R.G. and Chu, T.M. (1978) *J. Thor. Cardiovas. Surg.* 66:320). CEA was first described as a cancer specific fetal antigen in adenocarcinoma of the human digestive tract in 1965 (Gold, P. and Freeman, S.O. (1965) *Exp. Med.* 121:439). Since that time, CEA has been characterized as a cell surface antigen produced in excess in nearly all solid tumors of the human gastrointestinal tract. The gene for the human CEA protein has been cloned (Oikawa et al (1987) *Biochim. Biophys. Res.* 142:511-518; European Application No. EP 0346710). CEA is also expressed in fetal gut tissue and to a lesser extent on normal colon epithelium. The immunogenicity of CEA

Human carcinoembryonic antigen (CEA) is a 180 kD glycoprotein expressed on the majority of colon, rectal, stomach and pancreatic tumors (Muaro et al. (1985) *Cancer Res.* 45:5769), some 50% of breast carcinomas (Steward et al. (1974) *Cancer* 33:1246) and 70% of lung carcinomas (Vincent, R.G. and Chu, T.M. (1978) *J. Thor. Cardiovas. Surg.* 66:320). CEA was first described as a cancer specific fetal antigen in adenocarcinoma of the human digestive tract in 1965 (Gold, P. and Freeman, S.O. (1965) *Exp. Med.* 121:439). Since that time, CEA has been characterized as a cell surface antigen produced in excess in nearly all solid tumors of the human gastrointestinal tract. The gene for the human CEA protein has been cloned (Oikawa et al (1987) *Biochim. Biophys. Res.* 142:511-518; European Application No. EP 0346710). CEA is also expressed in fetal gut tissue and to a lesser extent on normal colon epithelium. The immunogenicity of CEA

has been ambiguous, with several studies reporting the presence of anti-CEA antibodies in patients (Gold et al. (1973) *Nature New Biology* 239:60; Pompecki, R. (1980) *Eur. J. Cancer* 16:973; Ura et al (1985) *Cancer Lett.*25:283; Fuchs et al. (1988) *Cancer Immunol. Immunother.* 26:180) while other studies have not (LoGerfo et al. (1972) *Int. J. Cancer* 9:344; MacSween, J.M. (1975) *Int. J. Cancer* 15:246; Chester K.A. and Begent, H.J. (1984) *Clin. Exp. Immunol.* 58:685).

Gp100 is normally found in melanosomes and expressed in melanocytes, retinal cells, and other neural crest derivatives. The function of gp100 is currently unknown. By mass spectrometry, three immunodominant HLA-A2 binding gp100 epitopes have been identified: g9-154 (amino acids 154-162), g9-209 (amino acids 209-217); and g9-280 (amino acids 280-288). Notably, two of these epitopes (as peptides) have been synthetically altered so as to induce a more vigorous immune response in the original T cell clone: the threonine at position 2 in gp-209 was changed to a methionine, and the alanine residue at position 9 in gp-280 was changed to a valine. These changes increase the binding affinity of the epitope-peptides to the HLA-A2 molecule without changing the intrinsic natural epitopes recognized by the T cell receptor (TCR). Rosenberg and colleagues (NIH) have already successfully immunized melanoma patients with one of these modified peptides and have reported achieving objective clinical responses in some patients.

Despite significant advances that have been made with respect to immunological approaches to cancer treatment, there is still a need in the art to improve cancer immunotherapies.

SUMMARY OF THE INVENTION

The present invention relates to improved methods for inducing and/or enhancing an immune response to a tumor antigen.

The present inventors have found that administering the tumor antigen or nucleic acid coding therefor directly into a lymphatic site (such as a lymph node) induces and/or significantly enhances the immune response to the tumor antigen and/or breaks tolerance to the tumor antigen, both
5 which have been a major challenge in previous methods of cancer immunotherapy.

Accordingly, one aspect the present invention provides a method for inducing and/or enhancing an immune response in an animal to a tumor antigen comprising administering an effective amount of a tumor antigen,
10 nucleic acid coding therefor, vector or cell comprising said nucleic acid, or vaccine comprising the aforementioned to a lymphatic site in the animal.

In another aspect, the present invention provides a method for breaking immune tolerance to a tumor antigen in an animal comprising administering an effective amount of a tumor antigen, nucleic acid coding
15 therefor, vector or cell comprising said nucleic acid, or vaccine comprising the aforementioned to a lymphatic site in the animal.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while
20 indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

25 The invention will now be described in relation to the drawings in which:

Figure 1 is a bar graph showing the results of an IFN- γ -ELISPOT analysis of an animal receiving an intranodal injection of the tumor antigen

Figure 2 is a bar graph showing the results of an IFN- γ -ELISPOT analysis of an animal receiving an intranodal injection of the tumor antigen

Figure 3 is a bar graph showing the results of an IFN- γ -ELISPOT analysis of an animal receiving a subcutaneous injection of the tumor antigen.

Figure 4 is a bar graph showing the results of an IFN- γ -ELISPOT analysis of an animal receiving a subcutaneous injection of the tumor antigen.

Figure 5 is a graph showing the antibody response after a regiment of intranodal (group 2) and subcutaneous (group 3) administration of ALVAC-modified gp100/modified gp100 peptide immunogens

Figure 6 is the nucleic acid sequence of a modified gp100M cDNA (SEQ.ID.NO.:109).

Figure 7 is the deduced amino acid sequence of the modified gp100M protein (SEQ.ID.NO.:110)

Figure 8 is the nucleic acid and amino acid sequence of a modified CEA (SEQ.ID.NOS.: 111 and 112).

DETAILED DESCRIPTION OF THE INVENTION

As hereinbefore mentioned, the present invention relates to an improved method for inducing and/or enhancing the immune response to a tumor antigen. Accordingly, the present invention provides a method for inducing and/or enhancing an immune response in an animal to a tumor antigen comprising administering an effective amount of a tumor antigen, a nucleic acid sequence encoding a tumor antigen, a vector or cell comprising the nucleic acid sequence, or a vaccine comprising the tumor antigen, the nucleic acid sequence encoding the tumor antigen, or a vector comprising the nucleic acid sequence encoding the tumor antigen to a lymphatic site in the animal.

The term "inducing and/or enhancing an immune response" means that the method evokes and/or enhances any response of the animal's immune system.

"Immune response" is defined as any response of the immune system, for example, of either a cell-mediated (i.e. cytotoxic T-lymphocyte mediated) or humoral (i.e. antibody mediated) nature. These immune responses can be assessed by a number of *in vivo* or *in vitro* assays well known to one skilled in the art including, but not limited to, antibody assays (for example ELISA assays) antigen specific cytotoxicity assays, production of cytokines (for example ELISPOT assays), regression of tumors expressing the tumor antigens, inhibition of cancer cells expressing the tumor antigens, etc..

The term "lymphatic site" means a site in the body that is associated with the lymphatic system including lymphatic organs, tissues, cells, nodes or glands such as spleen, thymus, tonsils, Peyer's patches, bone marrow, lymphocytes, thoracic duct as well as all of the lymph nodes of the body.

The term "animal" as used herein includes all members of the animal kingdom and is preferably human.

The term "effective amount" as used herein means an amount effective, at dosages and for periods of time necessary to achieve the desired results.

The term "tumor antigen" as used herein includes both tumor associated antigens (TAAs) and tumor specific antigens (TSAs). A tumor associated antigen means an antigen that is expressed on the surface of a tumor cell in higher amounts than is observed on normal cells or an antigen that is expressed on normal cells during fetal development. A tumor specific antigen is an antigen that is unique to tumor cells and is not expressed on normal cells. The term tumor antigen includes TAAs or TSAs

1. The first part of the paper is devoted to the study of the properties of the function $f(x)$ defined by the equation $f(x) = \int_0^x f(t) dt$. It is shown that the function $f(x)$ is continuous and differentiable on the interval $[0, 1]$. The derivative of $f(x)$ is equal to $f(x)$ itself. This implies that $f(x)$ is an exponential function. The initial condition $f(0) = 1$ determines the function uniquely as $f(x) = e^x$.

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(Fisk et al., *J. Exp. Med.*, 181:2109-2117 (1995)); Peoples et al., *Proc. Natl. Acad. Sci., USA*, 92:432-436 (1995)); mutated epidermal growth factor receptor (EGFR) (Fujimoto et al., *Eur. J. Gynecol. Oncol.*, 16:40-47 (1995)); Harris et al., *Breast Cancer Res. Treat.*, 29:1-2 (1994)); carcinoembryonic
 5 antigens (CEA) (Kwong et al., *J. Natl. Cancer Inst.*, 85:982-990 (1995)); carcinoma associated mutated mucins, for example, MUC-1 gene products (Jerome et al., *J. Immunol.*, 151:1654-1662 (1993), Ioannides et al., *J. Immunol.*, 151:3693-3703 (1993), Takahashi et al., *J. Immunol.*, 153:2102-2109 (1994)); EBNA gene products of EBV, for example, EBNA-1 gene
 10 product (Rickinson et al., *Cancer Surveys*, 13:53-80 (1992)); E7, E6 proteins of human papillomavirus (Ressing et al., *J. Immunol.*, 154:5934-5943 (1995)); prostate specific antigens (PSA) (Xue et al., *The Prostate*, 30:73-78 (1997)); prostate specific membrane antigen (PSMA) (Israeli, et al., *Cancer Res.*, 54:1807-1811 (1994)); PCTA-1 (Sue et al., *Proc. Natl. Acad. Sci. USA*,
 15 93:7252-7257 (1996)); idiotypic epitopes or antigens, for example, immunoglobulin idiotypes or T cell receptor idiotypes, (Chen et al., *J. Immunol.*, 153:4775-4787 (1994); Syrengelas et al., *Nat. Med.*, 2:1038-1040 (1996)); KSA (US Patent # 5348887); NY-ESO-1 (WO 98/14464).

Also included are modified tumor antigens and/or epitope/peptides
 20 derived therefrom (both unmodified and modified). Examples include, but are not limited to, modified and unmodified epitope/peptides derived from gp100 (WO 98/02598; WO 95/29193; WO 97/34613; WO 98/33810; CEA (WO 99/19478; S. Zaremba et al. (1997) *Cancer Research* 57:4570-7; K.T. Tsang et al. (1995) *J. Int. Cancer Inst.* 87:982-90); MART-1 (WO 98/58951,
 25 WO 98/02538; D. Valmeri et al. (2000) *J. Immunol.* 164:1125-31); p53 (M. Eura et al. (2000) *Clinical Cancer Research* 6:979-86); TRP-1 and TRP-2 (WO 97/29195); tyrosinase (WO 96/21734; WO 97/11669; WO 97/34613; WO 98/33810; WO 95/23234; WO 97/26535); KSA (WO 97/15597); PSA (WO

The experimental details and results are discussed in Example 1.

Additional embodiments of the invention encompass nucleic acid sequences comprising sequences encoding the tumor antigens and fragments or modified forms thereof as hereinbefore described. The term "nucleic acid sequence" refers to a sequence of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages. The term also includes modified or substituted sequences comprising non-naturally occurring monomers or portions thereof, which function similarly. The nucleic acid sequences of the present

invention may be ribonucleic (RNA) or deoxyribonucleic acids (DNA) and may contain naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The sequences may also contain modified bases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl, and
5 other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-amino adenine, 8-thiol adenine, 8-thio-alkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thioalkyl guanines, 8-hydroxyl guanine and other 8-
10 substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

The nucleic acid sequences encoding the tumor antigens of the invention include, but are not limited to, viral nucleic acid(s), plasmid(s), bacterial DNA, naked/free DNA and RNA. The nucleic acids encompass
15 both single and double stranded forms. As such, these nucleic acids comprise the relevant base sequences coding for the aforementioned tumor antigens. For purposes of definitiveness, the "relevant base sequences coding for the aforementioned polypeptides" further encompass complementary nucleic acid sequences. As such, embodiments of the
20 invention encompass nucleic acid sequences *per se* encoding for the aforementioned tumor antigens, or recombinant nucleic acids into which has been inserted said nucleic acids coding for tumor antigens (as described below).

Bacterial DNA useful in recombinant nucleic acid embodiments of the
25 invention are known to those of ordinary skill in the art. Sources of bacterial DNA include, for example, Shigella, Salmonella, Vibrio cholerae, Lactobacillus, Bacille Calmette Guérin (BCG), and Streptococcus. In bacterial DNA embodiments of the invention, nucleic acid of the invention may be inserted into the bacterial genome, can remain in a free state, or be
30 carried on a plasmid.

Viral recombinant nucleic acid embodiments of the invention may be derived from a poxvirus or other virus such as adenovirus or alphavirus.

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Preferably the viral nucleic acid is incapable of integration in recipient animal cells. The elements for expression from said nucleic acid may include a promoter suitable for expression in recipient animal cells.

Specific viral recombinant nucleic acid embodiments of the invention encompass (but are not limited to) poxviral, alphaviral, and adenoviral nucleic acid. Poxviral nucleic acid may be selected from the group consisting of avipox, orthopox, and suipox nucleic acid. Particular embodiments encompass poxviral nucleic acid selected from vaccinia, fowlpox, canary pox and swinepox; specific examples include TROVAC, NYVAC, ALVAC, MVA, Wyeth and Poxvac-TC (described in more detail below).

It is further contemplated that recombinant nucleic acids of this invention may further comprise nucleic acid sequences encoding at least one member chosen from the group consisting of cytokines, lymphokines, and co-stimulatory molecules. Examples include (but are not limited to) interleukin 2, interleukin 12, interleukin 6, interferon gamma, tumor necrosis factor Alpha, GM-CSF, B7.1, B7.2, ICAM-1, LFA-3, and Cd72.

Standard techniques of molecular biology for preparing and purifying nucleic acids well known to those skilled in the art can be used in the preparation of the recombinant nucleic acid aspects of the invention (for example, as taught in *Current Protocols in Molecular Biology*, F.M. Ausubel et al. (Eds.), John Wiley and Sons, Inc, N.Y., U.S.A. (1998), Chpts. 1, 2 and 4; *Molecular Cloning: A Laboratory Manual* (2nd Ed.), J. Sambrook, E.F. Fritsch and T. Maniatis (Eds.), Cold Spring Harbor Laboratory Press, N.Y., U.S.A. (1989), Chpts. 1, 2, 3 and 7).

Aspects of this invention further encompass vectors comprising the aforementioned nucleic acids. In certain embodiments, said vectors may be recombinant viruses or bacteria (as described below)

Adenovirus vectors and methods for their construction have been described (e.g. U.S. Patent Nos 5994132, 5932210, 6057158 and Published PCT Applications WO 9817783, WO 9744475, WO 9961034, WO 9950292, WO 9927101, WO 9720575, WO 9640955, WO 9630534-all of

5 9815636-all of which are herein incorporated by reference), as have lentivirus vectors (e.g. U.S. Patent Nos. 6013516, 5994136 and Published PCT Applications WO 9817816, WO 9712622, WO 9817815, WO 9839463, WO 9846083, WO 9915641, WO 9919501, WO 9930742, WO 9931251, WO 9851810, WO 0000600-all of which are herein incorporated by reference).

In one embodiment of the invention the poxvirus vector is ALVAC (1) or ALVAC (2) (both of which have been derived from canarypox virus). ALVAC (1) (or ALVAC (2)) does not productively replicate in non-avian hosts, a characteristic thought to improve its safety profile. ALVAC (1) is an attenuated canarypox virus-based vector that was a plaque-cloned derivative of the licensed canarypox vaccine, Kanapox (Tartaglia et al. (1992) *Virology* 188:217; U.S. Patent Nos. 5505941, 5756103 and 5833975-all of which are incorporated herein by reference). ALVAC (1) has some general properties which are the same as some general properties of Kanapox. ALVAC-based recombinant viruses expressing extrinsic immunogens have also been demonstrated efficacious as vaccine vectors (Tartaglia et al. In AIDS Research Reviews (vol. 3) Koff W., Wong-Staal F. and Kenedy R.C. (eds.), Marcel Dekker NY, pp. 361-378 (1993a), Tartaglia, J. et al. (1993b) *J. Virol.* 67:2370). For instance, mice immunized with an ALVAC (1) recombinant

expressing the rabies virus glycoprotein were protected from lethal challenge with rabies virus (Tartaglia, J et al., (1992) *supra*) demonstrating the potential for ALVAC (1) as a vaccine vector. ALVAC-based recombinants have also proven efficacious in dogs challenged with canine distemper virus (Taylor, J. et al (1992) *Virology* 187:321) and rabies virus (Perkus, M.E. et al., In Combined Vaccines and Simultaneous Administration: Current Issues and Perspective, Annals of the New York Academy of Sciences (1994)), in cats challenged with feline leukemia virus (Tartaglia, J. et al., (1993b) *supra*), and in horses challenged with equine influenza virus (Taylor, J. et al., In Proceedings of the Third International Symposium on Avian Influenza, Univ. of Wisconsin-Madison, Madison, Wisconsin, pp. 331-335 (1993)).

ALVAC (2) is a second-generation ALVAC vector in which vaccinia transcription elements E3L and K3L have been inserted within the C6 locus (U.S. Patent No. 5990091, incorporated herein by reference). The E3L encodes a protein capable of specifically binding to dsRNA. The K3L ORF has significant homology to E1F-2. Within ALVAC (2) the E3L gene is under the transcriptional control of its natural promoter, whereas K3L has been placed under the control of the early/late vaccine H6 promoter. The E3L and K3L genes act to inhibit PKR activity in cells infected with ALVAC (II), allowing enhancement of the level and persistence of foreign gene expression.

Additional viral vectors encompass natural host-restricted poxviruses. Fowlpox virus (FPV) is the prototypic virus of the Avipox genus of the Poxvirus family. Replication of avipox viruses is limited to avian species (Matthews, R.E.F. (1982) *Intervirology* 17:42) and there are no reports in the literature of avipox virus causing a productive infection in any non-avian species including man. This host restriction provides an inherent safety barrier to transmission of the virus to other species and makes use of avipox virus based vectors in veterinary and human applications an attractive proposition.

FPV has been used advantageously as a vector expressing immunogens from poultry pathogens. The hemagglutinin protein of a virulent avian influenza virus was expressed in an FPV recombinant. After inoculation of the recombinant into chickens and turkeys, an immune response was induced which was protective against either a homologous or a heterologous virulent influenza virus challenge (Taylor, J. et al. (1988) *Vaccine* 6: 504) FPV recombinants expressing the surface glycoproteins of Newcastle Disease Virus have also been developed (Taylor, J. et al. (1990) *J. Virol.* 64:1441; Edbauer, C. et al. (1990) *Virology* 179:901; U.S. Patent No. 5766599-incorporated herein by reference).

A highly attenuated strain of vaccinia, designated MVA, has also been used as a vector for poxvirus-based vaccines. Use of MVA is described in U.S. Patent No. 5,185,146.

Other attenuated poxvirus vectors have been prepared via genetic modification to wild type strains of vaccinia. The NYVAC vector, for example, is derived by deletion of specific virulence and host-range genes from the Copenhagen strain of vaccinia (Tartaglia, J. et al. (1992), *supra*; U.S. Patent Nos. 5364773 and 5494807-incorporated herein by reference) and has proven useful as a recombinant vector in eliciting a protective immune response against expressed foreign antigens.

Recombinant viruses can be constructed by processes known to those skilled in the art (for example, as previously described for vaccinia and avipox viruses; U.S. Patent Nos. 4769330; 4722848; 4603112; 5110587; and 5174993-all of which are incorporated herein by reference).

In further embodiments of the invention, live and/or attenuated bacteria may also be used as vectors. For example, non-toxicogenic *Vibrio cholerae* mutant strains may be useful as bacterial vectors in embodiments of this invention; as described in US Patent No. 4,882,278 (disclosing a strain in which a substantial amount of the coding sequence of each of the two *ctxA* alleles has been deleted so that no functional cholera toxin is produced), WO 92/11354 (strain in which the *irgA* locus is inactivated by mutation, this mutation can be combined in a single strain with *ctxA*

5 by reference.)

immunogens are described, for example, in WO 92/11361.

15 nucleic acid coding for a tumor antigen may be inserted into the bacterial genome, can remain in a free state, or be carried on a plasmid.

20 molecules. Said nucleic acid sequences can be contiguous with sequences coding for the tumor antigen or encoded on distinct nucleic acids.

transfection and/or infection into cells is dependant upon the intrinsic nature
of the introduced agent (i.e. free DNA, plasmid, recombinant virus), as will
be known to one skilled in the art (for example, as taught in *Current*

Protocols in Molecular Biology, F.M. Ausubel et al. (Eds.), John Wiley and Sons, Inc., N.Y., U.S.A. (1998), Chpt. 9; *Molecular Cloning: A Laboratory Manual (2nd Ed.)*, J. Sambrook, E.F. Fritsch and T. Maniatis (Eds.), Cold Spring Harbor Laboratory Press, N.Y., U.S.A. (1989), Chpts. 1, 2, 3 and 16).

- 5 Further embodiments of the invention encompass vaccines comprising the tumor antigens and/or nucleic acids coding therefor and/or vectors and/or cells previously described.

The vaccine of the invention comprising the tumor antigen may be a multivalent vaccine and additionally contain several peptides, epitopes or
10 fragments of a particular tumor antigen or contain peptides related to other tumor antigens and/or infectious agents in a prophylactically or therapeutically effective manner. Multivalent vaccines against cancers may contain a number of individual TAA's, or immunogenic fragments thereof, alone or in combinations which are effective to modulate an immune
15 response to cancer.

A vaccine of the invention may contain a nucleic acid molecule encoding a tumor antigen of the invention. Such vaccines are referred to as nucleic acid vaccines but are also termed genetic vaccines, polynucleotide vaccines or DNA vaccines, all of which are within the scope of the present
20 invention. In such an embodiment, the tumor antigen is produced *in vivo* in the host animal. Additional embodiments of the invention encompass vectors (i.e. bacteria, recombinant viruses) comprising the aforementioned nucleic acids.

The present invention also contemplates mixtures of the tumor
25 antigens, nucleic acids coding therefor, vectors comprising said nucleic acids, cells and/or vaccines comprising the aforementioned, and at least one member selected from the group consisting of cytokines, lymphokines, immunostimulatory molecules, and nucleic acids coding therefor. Additional embodiments of this invention further encompass
30 pharmaceutical compositions comprising the aforementioned tumor antigens, nucleic acids coding therefor, vectors, cells, vaccines or mixtures for administration to subjects in a biologically compatible form suitable for

administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention, or an "effective amount", is defined as an amount effective at dosages and for periods of time, necessary to achieve the desired result of eliciting an immune response in an animal. A therapeutically effective amount of a substance may vary according to factors such as the disease state/health, age, sex, and weight of the recipient, and the inherent ability of the particular tumor antigen, nucleic acid coding therefor, vector, cell, or vaccine to elicit a desired immune response. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, or at periodic intervals, and/or the dose may be proportionally reduced as indicated by the exigencies of circumstances.

The pharmaceutical compositions described herein can be prepared by *per se* known methods for the preparation of pharmaceutically acceptable compositions which can be administered to animals such that an effective quantity of the active substance (i.e. tumor antigen, nucleic acid, recombinant virus, vaccine) is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in "Handbook of Pharmaceutical Additives" (compiled by Michael and Irene Ash, Gower Publishing Limited, Aldershot, England (1995)). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and may be contained in buffered solutions with a suitable pH and/or be iso-osmotic with physiological fluids. In this regard, reference can be made to U.S. Patent No. 5,843,456. These compositions may further comprise an adjuvant (as described below).

Further embodiments of the invention encompass methods of inhibiting a tumor antigen expressing cancer cell in a patient comprising administering to said patient an effective amount of a tumor antigen, nucleic

acid coding therefor, vector, cell, or vaccine of the invention. Patients with solid tumors expressing tumor antigens include (but are not limited to) those suffering from colon cancer, lung cancer, pancreas cancer, endometrial cancer, breast cancer, thyroid cancer, melanoma, oral cancer, laryngeal cancer, seminoma, hepatocellular cancer, bile duct cancer, squamous cell carcinoma, and prostate cancer. As such, methods of treating patients with cancer *per se* encompassing the aforementioned methods of inducing an immune response and/or inhibiting a tumor antigen expressing cell are contemplated aspects/embodiments of the invention.

As mentioned previously, an animal may be immunized with a tumor antigen, nucleic acid coding therefore, vector, cell or vaccine of the invention by administering the aforementioned to a lymphatic site. The administration can be achieved in a single dose or repeated at intervals. The appropriate dosage is dependant on various parameters understood by the skilled artisans, such as the immunogen itself (i.e. polypeptide vs. nucleic acid (and more specifically type thereof)), the route of administration and the condition of the animal to be vaccinated (weight, age and the like).

As previously noted, nucleic acids (in particular plasmids and/or free/naked DNA and/or RNA coding for the tumor antigen of the invention) can be administered to an animal for purposes of inducing/eliciting an immune response (for example, US Patent No. 5589466; McDonnell and Askari, *NEJM* 334:42-45 (1996); Kowalczyk and Ertl, *Cell Mol. Life Sci.* 55:751-770 (1999)). Typically, this nucleic acid is a form that is unable to replicate in the target animal's cell and unable to integrate in said animal's genome. The DNA/RNA molecule encoding the tumor antigen is also typically placed under the control of a promoter suitable for expression in the animal's cell. The promoter can function ubiquitously or tissue-specifically. Examples of non-tissue specific promoters include the early Cytomegalovirus (CMV) promoter (described in U.S. Patent No. 4,168,062) and the Rous Sarcoma Virus promoter. The desmin promoter is tissue-specific and drives expression in muscle cells. More generally, useful vectors have been described (i.e., WO 94/21797).

For administration of nucleic acids coding for a tumor antigen, said nucleic acid can encode a precursor or mature form of the polypeptide/protein. When it encodes a precursor form, the precursor form can be homologous or heterologous. In the latter case, a eucaryotic leader sequence can be used, such as the leader sequence of the tissue-type plasminogen factor (tPA).

For use as an immunogen, a nucleic acid of the invention can be formulated according to various methods known to a skilled artisan. First, a nucleic acid can be used in a naked/free form, free of any delivery vehicles (such as anionic liposomes, cationic lipids, microparticles, (e.g., gold microparticles), precipitating agents (e.g., calcium phosphate) or any other transfection-facilitating agent. In this case the nucleic acid can be simply diluted in a physiologically acceptable solution (such as sterile saline or sterile buffered saline) with or without a carrier. When present, the carrier preferably is isotonic, hypotonic, or weakly hypertonic, and has a relatively low ionic strength (such as provided by a sucrose solution (e.g., a solution containing 20% sucrose)).

Alternatively, a nucleic acid can be associated with agents that assist in cellular uptake. It can be, i.e., (i) complemented with a chemical agent that modifies the cellular permeability (such as bupivacaine; see, for example, WO 94/16737), (ii) encapsulated into liposomes, or (iii) associated with cationic lipids or silica, gold, or tungsten microparticles.

Cationic lipids are well known in the art and are commonly used for gene delivery. Such lipids include Lipofectin (also known as DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride), DOTAP (1,2-bis(oleyloxy)-3-(trimethylammonio) propane), DDAB (dimethyldioctadecylammonium bromide), DOGS (dioctadecylamidoglycyl spermine) and cholesterol derivatives such as DC-Chol (3 beta-(N-(N',N'-dimethyl aminomethane)-carbamoyl) cholesterol). A description of these cationic lipids can be found in EP 187702, WO 90/11092, U.S. Patent No. 5283185, WO 91/15501, WO 95/26356, and U.S. Patent No. 5527928. Cationic lipids for gene delivery are preferably used in association with a neutral lipid such

as DOPE (dioleoyl phosphatidylethanolamine) as, for example, described in WO 90/11092.

Other transfection-facilitating compounds can be added to a formulation containing cationic liposomes. A number of them are described in, for example, WO 93/18759, WO 93/19768, WO 94/25608, and WO 95/2397. They include, for example, spermine derivatives useful for facilitating the transport of DNA through the nuclear membrane (see, for example, WO 93/18759) and membrane-permeabilizing compounds such as GALA, Gramicidine S, and cationic bile salts (see, for example, WO 93/19768).

Gold or tungsten microparticles can also be used for nucleic acid delivery (as described in WO 91/359 and WO 93/17706). In this case, the microparticle-coated polynucleotides can be injected via intradermal or intraepidermal routes using a needleless injection device ("gene gun"; such as those described, for example, in U.S. Patent No. 4,945,050, U.S. Patent No. 5,015,580, and WO 94/24263).

Anionic and neutral liposomes are also well-known in the art (see, for example, *Liposomes: A Practical Approach*, RPC New Ed, IRL Press (1990), for a detailed description of methods for making liposomes) and are useful for delivering a large range of products, including nucleic acids.

Particular embodiments of the aforementioned methods (i.e. to induce/elicit immune responses) encompass prime-boost protocols for the administration of immunogens of the invention. More specifically, these protocols encompass (but are not limited to) a "priming" step with a particular/distinct form of immunogen (i.e. nucleic acid (for example, plasmid, bacterial/viral/free or naked)) coding for tumor antigen, or vector (i.e. recombinant virus, bacteria) comprising said nucleic acid) followed by at least one "boosting" step encompassing the administration of an alternate (i.e. distinct from that used to "prime") form of the tumor antigen (i.e. protein or fragment thereof (for example, epitope/peptide), nucleic acid coding for the tumor antigen (or fragment thereof), or vector comprising said nucleic acid). Examples of "prime-boost" methodologies are known to

those skilled in the art (as taught, for example, in PCT published applications WO 98/58956, WO 98/56919, WO 97/39771). One advantage of said protocols is the potential to circumvent the problem of generating neutralizing immune responses to vectors *per se* (i.e. recombinant viruses) wherein is inserted/incorporated nucleic acids encoding the immunogen or fragments thereof (see for example, R.M. Conry et al (2000) *Clin. Cancer Res.* 6.34-41).

As is well known to those of ordinary skill in the art, the ability of an immunogen to induce/elicit an immune response can be improved if, regardless of administration formulation (i.e. recombinant virus, nucleic acid, polypeptide), said immunogen is co-administered with an adjuvant. Adjuvants are described and discussed in "Vaccine Design-the Subunit and Adjuvant Approach" (edited by Powell and Newman, Plenum Press, New York, U.S.A., pp. 61-79 and 141-228 (1995)). Adjuvants typically enhance the immunogenicity of an immunogen but are not necessarily immunogenic in and of themselves. Adjuvants may act by retaining the immunogen locally near the site of administration to produce a depot effect facilitating a slow, sustained release of immunizing agent to cells of the immune system. Adjuvants can also attract cells of the immune system to an immunogen depot and stimulate such cells to elicit immune responses. As such, embodiments of this invention encompass compositions further comprising adjuvants.

Desirable characteristics of ideal adjuvants include:

- 1) lack of toxicity;
- 2) ability to stimulate a long-lasting immune response;
- 3) simplicity of manufacture and stability in long-term storage;
- 4) ability to elicit both cellular and humoral responses to antigens administered by various routes, if required;
- 5) synergy with other adjuvants;
- 6) capability of selectively interacting with populations of antigen presenting cells (APC);

- 7) ability to specifically elicit appropriate TH1 or TH2 cell-specific immune responses; and
- 8) ability to selectively increase appropriate antibody isotype levels (for example, IgA) against antigens/immunogens.

5 However, many adjuvants are toxic and can cause undesirable side effects, thus making them unsuitable for use in humans and many animals. For example, some adjuvants may induce granulomas, acute and chronic inflammations (i.e. Freund's complete adjuvant (FCA)), cytotoxicity (i.e. saponins and pluronic polymers) and pyrogenicity, arthritis and anterior
10 uveitis (i.e. muramyl dipeptide (MDP) and lipopolysaccharide (LPS)). Indeed, only aluminum hydroxide and aluminum phosphate (collectively commonly referred to as alum) are routinely used as adjuvants in human and veterinary vaccines. The efficacy of alum in increasing antibody responses to diphtheria and tetanus toxoids is well established.

15 Notwithstanding, it does have limitations. For example, alum is ineffective for influenza vaccination and inconsistently elicits a cell mediated immune response with other immunogens. The antibodies elicited by alum-adjuvanted antigens are mainly of the IgG1 isotype in the mouse, which may not be optimal for protection in vaccination contexts.

20 Adjuvants may be characterized as "intrinsic" or "extrinsic". Intrinsic adjuvants (such as lipopolysaccharides) are integral and normal components of agents which in themselves are used as vaccines (i.e. killed or attenuated bacteria). Extrinsic adjuvants are typically nonintegral immunomodulators generally linked to antigens in a noncovalent manner,
25 and are formulated to enhance the host immune response

 In embodiments of the invention, adjuvants can be at least one member chosen from the group consisting of cytokines, lymphokines, and co-stimulatory molecules. Examples include (but are not limited to)

5 5759535, 5254534, European Patent Application EP 211684, and published
PCT document WO 97/28816 - all of which are herein incorporated by
reference).

10 antigens (immune stimulating complexes), pluronic polymers with mineral oil, killed mycobacteria and mineral oil, Freund's complete adjuvant, bacterial products such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as well as lipid A, and liposomes.

15 (Lacaille-Dubois, M. and Wagner, H. (1996) *Phytomedicine* 2:363). For example, Quil A (derived from the bark of the South American tree *Quillaja Saponaria* Molina) and fractions thereof has been extensively described (i.e. U.S. Patent No. 5057540; Kensil, C.R. (1996) *Crit Rev Ther Drug Carrier Syst.* 12:1; and European Patent EP 362279). The haemolytic saponins
20 QS21 and QS17 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants (U.S. Patent No. 5057540; European Patent EP 362279). Also described in these references is the use of QS7 (a non-haemolytic fraction of Quil-A) which acts as a potent adjuvant for systemic vaccines. Use of QS21 is further described in Kensil et al. ((1991) *J.*
25 *Immunol* 146:431). Combinations of QS21 and polysorbate or cyclodextrin are also known (WO 9910008). Particulate adjuvant systems comprising fractions of Quil A (such as QS21 and QS7) are described in WO 9633739 and WO 9611711

Another preferred adjuvant/immunostimulant is an immunostimulatory oligonucleotide containing unmethylated CpG dinucleotides ("CpG"). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. CpG is known in the art as being an adjuvant when administered by both systemic and mucosal routes (WO 9602555; European Patent EP 468520; Davies et al (1998) *J. Immunol.* 160:87; McCluskie and Davis (1998) *J. Immunol.* 161:4463) In a number of studies, synthetic oligonucleotides derived from BCG gene sequences have also been shown to be capable of inducing immunostimulatory effects (both in vitro and in vivo; Krieg, (1995) *Nature* 374:546). Detailed analyses of immunostimulatory oligonucleotide sequences has demonstrated that the CG motif must be in a certain sequence context, and that such sequences are common in bacterial DNA but are rare in vertebrate DNA. (For example, the immunostimulatory sequence is often: purine, purine, C, G, pyrimidine, pyrimidine, wherein the CG motif is not methylated; however other unmethylated CpG sequences are known to be immunostimulatory and as such may also be used in the present invention.) As will be evident to one of normal skill in the art, said CG motifs/sequences can be incorporated into nucleic acids of the invention *per se*, or reside on distinct nucleic acids.

A variety of other adjuvants are taught in the art, and as such are encompassed by embodiments of this invention. U.S. Patent No. 4,855,283 granted to Lockhoff et al. (incorporated herein by reference) teaches glycolipid analogues and their use as adjuvants. These include N-glycosylamides, N-glycosylureas and N-glycosylcarbamates, each of which is substituted in the sugar residue by an amino acid, as immunomodulators or adjuvants. Furthermore, Lockhoff et al. ((1991) *Chem. Int. Ed. Engl.* 30:1611) have reported that N-glycolipid analogs displaying structural similarities to the naturally-occurring glycolipids (such as

[illegible][illegible][illegible][illegible]

In further aspects of this invention, adjuvants useful for parenteral administration of immunizing agent include aluminum compounds (such as aluminum hydroxide, aluminum phosphate, and aluminum hydroxy phosphate; but might also be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes). The antigen can be precipitated with, or adsorbed onto, the aluminum compound according to standard protocols well known to those skilled in the art.

Adjuvants for mucosal immunization may include bacterial toxins (e.g., the cholera toxin (CT), the *E. coli* heat-labile toxin (LT), the *Clostridium difficile* toxin A and the pertussis toxin (PT), or combinations, subunits, toxoids, or mutants thereof). For example, a purified preparation of native cholera toxin subunit B (CTB) can be of use. Fragments, homologs, derivatives, and fusion to any of these toxins are also suitable, provided that they retain adjuvant activity. A mutant having reduced toxicity may be used. Mutants have been described (e.g., in WO 95/17211 (Arg-7-Lys CT mutant), WO 96/6627 (Arg-192-Gly LT mutant), and WO 95/34323 (Arg-9-Lys and Glu-129-Gly PT mutant)). Additional LT mutants include, for example Ser-63-Lys, Ala-69-Gly, Glu-110-Asp, and Glu-112-Asp mutants. Other adjuvants (such as a bacterial monophosphoryl lipid A (MPLA)) of various sources (e.g., *E. coli*, *Salmonella minnesota*, *Salmonella typhimurium*, or *Shigella flexneri*) can also be used in the mucosal administration of immunizing agents.

Adjuvants/immunostimulants as described herein may be formulated together with carriers, such as for example liposomes, oil in water emulsions, and/or metallic salts including aluminum salts (such as

aluminum hydroxide). For example, 3D-MPL may be formulated with aluminum hydroxide (as discussed in EP 689454) or oil in water emulsions (as discussed in WO 9517210); QS21 may be advantageously formulated with cholesterol containing liposomes (as discussed in WO 9633739), in oil
 5 water emulsions (as discussed in WO 9517210) or alum (as discussed in WO 9815287). When formulated into vaccines, immunostimulatory oligonucleotides (i.e. CpGs) are generally administered in free solution together with free antigen (as discussed in WO 9602555; McCluskie and Davis (1998) *Supra*), covalently conjugated to an antigen (as discussed in
 10 WO 9816247), or formulated with a carrier such as aluminum hydroxide or alum (as discussed in Davies et al. *Supra*; Brazolot-Millan et al (1998) *Proc. Natl. Acad. Sci.* 95:15553).

Combinations of adjuvants/immunostimulants are also within the scope of this invention. For example, a combination of a monophosphoryl
 15 lipid A and a saponin derivative (as described in WO 9400153, WO 9517210, WO 9633739, WO 9856414, WO 9912565, WO 9911214) can be used, or more particularly the combination of QS21 and 3D-MPL (as described in WO 9400153). A combination of an immunostimulatory oligonucleotide and a saponin (such as QS21), or a combination of
 20 monophosphoryl lipid A (preferably 3D-MPL) in combination with an aluminum salt also form a potent adjuvant for use in the present invention.

The following non-limiting example is illustrative of the present invention:

EXAMPLES

25 Example 1

This example compares the intranodal injection with subcutaneous injection of a representative tumor antigen (modified gp100).

Methods and Experimental Design

Test System

Cynomolgus monkeys (Macaca fascicularis) purpose bred animals.

Supplier: Siconbrec "Simian Conservation Breeding & Research Center
5 Inc.", Fema Building, 44 Gil Puyat Avenue Makati, Metro Manila, Philippines.

Number of animals in the study: 12 (6 males and 6 females).

Age at initiation of treatment: 26 to 38 months.

- Body weight range at initiation of treatment (day -1)
- males: 1.73 to 2.34 kg
- 10 ▪ females: 1.71 to 2.65 kg.

Animal Husbandry

- Housing: one air-conditioned room;
- temperature: 19 to 25°C (target range),
- relative humidity: >40%
- 15 ▪ air changes: minimum 8 air changes per hour,
- lighting cycle: 12 hours light (artificial)/12 hours dark.
- Caging: animals were housed singly in stainless steel mesh cages
(approximately 540 x 810 x 760 mm).
- Diet: expanded complete commercial primate diet (Mazuri diet, Special
20 Diet Services Ltd., Witham, Essex, CM8, 3AD, Great Britain) analyzed for
chemical and bacterial contaminants.

Quantity distributed: 100g diet/animal/day.

In addition, animals received fruit daily (apple or banana)

Animals were fasted for at least 16 hours before blood sampling for clinical
25 laboratory investigations and before necropsy.

- Water: drinking water *ad libitum* (via bottles)
- Contaminants: no known contaminants were present in diet or water at
levels which might have interfered with achieving the objective of the study.

Pre-Treatment Procedures

- Animal health procedure: all animals received a clinical examination for ill-health on arrival and a veterinary clinical examination during the acclimatization period.
- 5 ▪ Acclimatization period: at least 3 weeks between animal arrival and start of treatment.

Experimental Design

- Allocation to treatment groups was performed during the acclimatization period using a random allocation procedure based on body weight classes.
- 10 ▪ Animals were assigned to the treatment groups shown in Table 1. The dose levels administered were shown in Table 2.

Administration of the Test/Control Articles

Group 1 and 2 Animals

- Method of administration: injection in the left inguinal lymph node.
- 15 Animals were lightly anaesthetized before each administration by an intramuscular injection of ketmine hydrochloride (Imalgene® 500 - Merial, Lyon, France). The same lymph node was injected on each occasion (left side). Each injection was followed by a local disinfection with iodine (Vétédine® - Vétoquinol, Lure, France).

20 Group 3

- Route: subcutaneous.
 - Method of administration: bolus injection using a sterile syringe and needle introduced subcutaneously. Four injection sites were used followed by a local disinfection with iodine (Vétédine® - Vétoquinol, Lure, France).
- 25 Animals were also lightly anaesthetized before each administration by an intramuscular injection of ketamine hydrochloride (Imalgene® 500 - Merial, Lyon, France) in order to be under the same conditions as groups 1 and 2 animals.

stimulus (PMA-Iono) The pooled peptides used in this Example to stimulate IFN- γ production were derived from gp100 and are illustrated in Tables 4 to 7. The final volume of each sample is 200 μ L. Incubate 20 hours at 37°C.

- 5 Wash 4 times in 1X PBS and 0.05% Tween 20.

Detection

Place 100 μ L per well of detection antibodies at 1 μ g/mL diluted in 1/1000 1X PBS, 1% BSA and 0.05% Tween 20. Incubate 2 hours at room temperature. Wash 4 times in 1X PBS and 0.05% Tween 20.

- 10 **Reaction**

Place 100 μ L per well of Extravidin-PA conjugate diluted 1/6000 in 1X PBS, 1% BSA and 0.05% Tween 20. Incubate 45 minutes at room temperature.

Wash 4 times in 1X PBS and 0.05% Tween 20.

Substrate Addition

- 15 Place 100 μ L per well of substrate previously prepared. For example, for 1 plate, prepare 9.6 mL of distilled water, 0.4 mL of 25X buffer, 0.1 mL of solution A (NBT) and 0.1 mL of solution B (BCIP). Incubate 30-45 minutes at room temperature. Wash in distilled water. Dry and transfer to a plastic film. The number of spots are counted using a Zeiss image analyzer. Each
20 spot corresponds to an individual IFN- γ secreting T cell.

Results

- The animals that tested positive on the ELISPOT analysis are shown in Figures 1-4. Overall, the results demonstrate that of the animals tested, 2
25 out of 2 (i.e. 100%) of the animals that received the intranodal administration of the gp100 antigen, and 2 out of 4 (i.e. 50%) of the animals that received the subcutaneous administration of the gp100 antigen had a positive cell mediated immune response.

ELISA Analysis

The ELISA was performed utilizing standard methodology known in the art. Briefly, the human gp100 ("hgp100"; produced in Baculovirus) was diluted in coating buffer (carbonate-bicarbonate, pH9.6) and added to 96 wells at 0.5ug/well. Plates were placed at 4°C overnight. Plates were then washed and blocking buffer (phosphate buffered saline/0.5% Tween 20/1.0% BSA, pH7.2) was added for 2 hours at 37°C. The plates were then washed and the sera was diluted in dilution buffer (phosphate buffered saline/0.5 % Tween 20/ 0.1 BSA, pH7.2). For this study, monkey sera was diluted to 1:800 and "7" serial 3 fold dilutions were done for each sample tested. The human sera controls were diluted to 1:50 in dilution buffer and "7" serial 2 fold dilutions were performed. Each dilution was done in duplicate. The plates were incubated a further 2 hours at 37°C. The plates were washed and the horse radish peroxidase (HRP)-conjugated anti-human secondary antibody (anti-human Ig whole antibody from sheep (Amersham Life Science, NA933)) diluted 1:100 in dilution buffer was added to the wells and incubated for 1 hour at 37°C. The plates were washed and OPD (o-phenylenediamine dihydrochloride) substrate with H₂O₂ in substrate buffer (50mM phosphate/25mM citrate, pH 7.2) was added to the wells. For a kinetics ELISA, the plate was read repeatedly (2 minute intervals for 15 minutes) unstopped (without "stop" buffer). Plates were read at 450nm.

Results

The results of the above experiment are presented in Table 8 and in Figure 5. The animals of group 2 received intranodal injections of ALVAC(2)-gp100(mod) followed by boosts with the modified gp100 peptides 209(2M) and 290(9V); the animals in group 3 received a subcutaneous

TABLE 1

Group Number	Route of administration	Treatment days and compound administered	Number of Animals
1	Intranodal	Saline (NaCl 0.9%): days 28, 42, 56 Then 70, 71, 72, 73, 74 Then 84, 85, 86, 87 and 88	4
2	Intranodal	ALVAC(2) - gp100 mod days 28, 42, 56 *mcp100 peptides days 70, 71, 72, 73, 74 Then 84, 85, 86, 87 and 88	4
3	Subcutaneous	Saline (NaCl 0.9%): day 1 ALVAC(2) - gp100 mod days 28, 42, 56 *mcp100 peptides: days 70 and 84	4

*209(2M)-IMDQVPFSY, 290(9V) YLEPGPVTY

- 5
- Group 1 animals (control) received the control article (saline for injection (NaCl 0.9%)).
 - Group 3 animals received the control article (saline for injection (NaCl 0.9%)) on day 1 only

36
TABLE 2

Group Number	Dose level	Dose volume (ml/administration)
1	Saline (NaCl 0.9%): 0	0.250
2	Dose: $0.25 \times 10^{7.5}$ CCID ₅₀ ALVAC (2) - gp100 mod: $0.25 \times 10^{7.5}$ CCID ₅₀ Dose: 200 µg (Total) of peptides IMDQVPFSY (209(2M)), and YLEPGPVTV (290(9V)) (100µg each)	0.250 0.2
3	Saline (NaCl 0.9%) ALVAC(2) - gp100 mod: $0.25 \times 10^{7.5}$ CCID ₅₀ Dose: 200 µg (Total) of peptides IMDQVPFSY (209(2M)), and YLEPGPVTV (290(9V)) (100µg each)	0.250 0.250 0.2

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Days	Sites used
1 and 28	lower left
42	upper left
56	upper right
70	lower left
84	lower right

$\frac{d^2\psi}{dx^2} + \frac{d\psi}{dx} + \psi = 0$

Peptide Pool #1

Peptide	Sequence	SEQ.ID.NO.
1329	HLAVIGALLAVGATK	SEQ.ID.NO.3
1330	GALLAVGATKVPRNQ	SEQ.ID.NO.4
1331	VGATKVPRNQDWLGV	SEQ.ID.NO.5
1332	VPRNQDWLGVSRQLR	SEQ.ID.NO.6
1333	DWLGVSRQLRTKAWN	SEQ.ID.NO.7
1334	SRQLRTKAWNROLYP	SEQ.ID.NO.8
1335	TKAWNROLYPEWTEA	SEQ.ID.NO.9
1336	ROLYPEWTEAQRLLDC	SEQ.ID.NO.10
1337	EWTEAQRLLDCWRGGQ	SEQ.ID.NO.11
1338	QRLLDCWRGGQVSLKV	SEQ.ID.NO.12
1339	WRGGQVSLKVSNDGP	SEQ.ID.NO.13
1340	VSLKVSNDGPTLIGA	SEQ.ID.NO.14
1344	IALNFPQSQKVLPGD	SEQ.ID.NO.15
1345	PGSQKVLPGDQVIWV	SEQ.ID.NO.16
1346	VLPGDQVIWVNNTH	SEQ.ID.NO.17
1347	QVIWVNNTHINGSQV	SEQ.ID.NO.18
1348	NNTHINGSQVWGGQP	SEQ.ID.NO.19
1349	NGSQVWGGQPVYPQE	SEQ.ID.NO.20
1350	WGGQPVYPQETDDAC	SEQ.ID.NO.21
1351	VYPQETDDACIFPDG	SEQ.ID.NO.22
1352	TDDACIFPDGGPCPS	SEQ.ID.NO.23
1353	IFPDGGPCPSGSWSQ	SEQ.ID.NO.24
1355	GSWSQKRSFVYVWKT	SEQ.ID.NO.25
1356	KRSFVYVWKTWGQYW	SEQ.ID.NO.26
1357	YVWKTWGQYWQVLGG	SEQ.ID.NO.27
1358	WGQYWQVLGGPVSGL	SEQ.ID.NO.28
1359	QVLGGPVSGLSIGTG	SEQ.ID.NO.29

TABLE 5

Peptide Pool #2

Peptide	Sequence	SEQ.ID.NO.
1360	PVSGLSIGTGRAMLG	SEQ.ID.NO.30
1361	SIGTGRAMLGHTTME	SEQ.ID.NO.31
1362	RAMLGHTTMEVTVYH	SEQ.ID.NO.32
1363	THTMEVTVYHRRGSR	SEQ.ID.NO.33
1364	VTVYHRRGSRSYVPI	SEQ.ID.NO.34
1365	RRGSRSYVPLAHSSS	SEQ.ID.NO.35
1366	SYVPLAHSSSAFTIT	SEQ.ID.NO.36
1368	AFTITDQVPFSVS	SEQ.ID.NO.37
1369	DQVPFSVSVSQRLAL	SEQ.ID.NO.38
1370	SVSVSQRLALDGGNK	SEQ.ID.NO.39
1372	DGGNKHFLRNQPLTF	SEQ.ID.NO.40
1373	HFLRNQPLTFALQLH	SEQ.ID.NO.41
1374	QPLTFALQLHDPSTGY	SEQ.ID.NO.42
1375	ALQLHDPSTGYLAED	SEQ.ID.NO.43
1379	DFGDSSGTLISRALV	SEQ.ID.NO.44
1380	STGLISRALVVTHTY	SEQ.ID.NO.45
1381	SRALVVTHTYLEPGP	SEQ.ID.NO.46
1382	VTHTYLEPGPVTAQV	SEQ.ID.NO.47
1383	LEPGPVTAQVVLQAA	SEQ.ID.NO.48
1384	VTAQVVLQAAIPLTS	SEQ.ID.NO.49
1385	VLQAAIPLTSCGSSP	SEQ.ID.NO.50
1386	IPLTSCGSSPVPGETT	SEQ.ID.NO.51
1388	VPGETTDGHRPTAEAP	SEQ.ID.NO.52
1389	DGHRPTAEAPNTTAG	SEQ.ID.NO.53
1390	TAEAPNTTAGQVPTT	SEQ.ID.NO.54
1392	QVPTTEVVGTTPGQA	SEQ.ID.NO.55
1393	EVVGTTTPGQAPTAEP	SEQ.ID.NO.56

Peptide	Sequence	SEQ.ID.NO.
1394	TPGQAPTAEPSGTTT	SEQ.ID.NO.57
1395	PTAEPSGTTT VQVPT	SEQ.ID.NO.58
1396	SGTTT VQVPTTEVIS	SEQ.ID.NO.59
1397	VQVPTTEVISTAPVQ	SEQ.ID.NO.60
1398	TEVISTAPVQMPTAE	SEQ.ID.NO.61
1399	TAPVQMPTAESTGMT	SEQ.ID.NO.62
1400	MPTAESTGMTPEKVP	SEQ.ID.NO.63
1401	STGMTPEKVPVSEVM	SEQ.ID.NO.64
1402	PEKVPVSEVMGTTLA	SEQ.ID.NO.65
1403	VSEVMGTTLAEMSTP	SEQ.ID.NO.66
1404	GTTLAEMSTPEATGM	SEQ.ID.NO.67
1405	EMSTPEATGMTPAEV	SEQ.ID.NO.68
1408	SIVVLSGTTAAQVTT	SEQ.ID.NO.69
1409	SGTTAAQVTTTEWVE	SEQ.ID.NO.70
1410	AQVTTTEWVETTARE	SEQ.ID.NO.71
1411	TEWVETARELPIPE	SEQ.ID.NO.72
1412	TTARELPIPEPEGPD	SEQ.ID.NO.73
1413	LPIPEPEGPDASSIM	SEQ.ID.NO.74
1414	PEGPDASSIMSTESI	SEQ.ID.NO.75
1415	ASSIMSTESITGSLG	SEQ.ID.NO.76
1416	STESITGSLGPLLDG	SEQ.ID.NO.77
1417	TGSLGPLLDGTATLR	SEQ.ID.NO.78
1418	PLLDGTATLRLVKRQ	SEQ.ID.NO.79
1419	TATLRLVKRQVPLDC	SEQ.ID.NO.80
1420	LVKRQVPLDCVLYRY	SEQ.ID.NO.81
1421	VPLDCVLYRYGSFSV	SEQ.ID.NO.82
1422	VLYRYGSFSVTLDIV	SEQ.ID.NO.83

Peptide	Sequence	SEQ.ID.NO.
1394	TPGQAPTAEPSGTTT	SEQ.ID.NO.57
1395	PTAEPSGTTT VQVPT	SEQ.ID.NO.58
1396	SGTTT VQVPTTEVIS	SEQ.ID.NO.59
1397	VQVPTTEVISTAPVQ	SEQ.ID.NO.60
1398	TEVISTAPVQMPTAE	SEQ.ID.NO.61
1399	TAPVQMPTAESTGMT	SEQ.ID.NO.62
1400	MPTAESTGMTPEKVP	SEQ.ID.NO.63
1401	STGMTPEKVPVSEVM	SEQ.ID.NO.64
1402	PEKVPVSEVMGTTLA	SEQ.ID.NO.65
1403	VSEVMGTTLAEMSTP	SEQ.ID.NO.66
1404	GTTLAEMSTPEATGM	SEQ.ID.NO.67
1405	EMSTPEATGMTPAEV	SEQ.ID.NO.68
1408	SIVVLSGTTAAQVTT	SEQ.ID.NO.69
1409	SGTTAAQVTTTEWVE	SEQ.ID.NO.70
1410	AQVTTTEWVETTARE	SEQ.ID.NO.71
1411	TEWVETARELPIPE	SEQ.ID.NO.72
1412	TTARELPIPEPEGPD	SEQ.ID.NO.73
1413	LPIPEPEGPDASSIM	SEQ.ID.NO.74
1414	PEGPDASSIMSTESI	SEQ.ID.NO.75
1415	ASSIMSTESITGSLG	SEQ.ID.NO.76
1416	STESITGSLGPLLDG	SEQ.ID.NO.77
1417	TGSLGPLLDGTATLR	SEQ.ID.NO.78
1418	PLLDGTATLRLVKRQ	SEQ.ID.NO.79
1419	TATLRLVKRQVPLDC	SEQ.ID.NO.80
1420	LVKRQVPLDCVLYRY	SEQ.ID.NO.81
1421	VPLDCVLYRYGSFSV	SEQ.ID.NO.82
1422	VLYRYGSFSVTLDIV	SEQ.ID.NO.83

Peptide	Sequence	SEQ.ID.NO.
1424	TLDIVQIGESAEILQ	SEQ.ID.NO.84
1425	QIGESAEILQAVPSG	SEQ.ID.NO.85
1426	AEILQAVPSGEGDAF	SEQ.ID.NO.86
1427	AVPSGEGDAFELTVS	SEQ.ID.NO.87
1428	EGDAFELTVSCQGGL	SEQ.ID.NO.88
1429	ELTVSCQGGPLKEAC	SEQ.ID.NO.89
1430	CQGGLPKEACMEISS	SEQ.ID.NO.90
1431	PKEACMEISSPGCQP	SEQ.ID.NO.91
1432	MEISSPGCQPPAQR	SEQ.ID.NO.92
1434	PAQRLCQPVLPSPAC	SEQ.ID.NO.93
1435	CQPVLPSPACQLVLH	SEQ.ID.NO.94
1436	PSPACQLVLHQILKG	SEQ.ID.NO.95
1437	QLVLHQILKGGSGTY	SEQ.ID.NO.96
1441	LADTNSLAVVSTQLI	SEQ.ID.NO.97
1442	SLAVVSTQLIMPGQE	SEQ.ID.NO.98
1443	STQLIMPGQEAGLGQ	SEQ.ID.NO.99
1444	MPGQEAGLGQVPLIV	SEQ.ID.NO.100
1445	AGLGQVPLIVGILLV	SEQ.ID.NO.101
1448	LMAVVLASLIYRRRL	SEQ.ID.NO.102
1450	YRRRLMKQDFSVPQL	SEQ.ID.NO.103
1451	MKQDFSVPQLPHSSS	SEQ.ID.NO.104
1452	SVPQLPHSSSHWLRL	SEQ.ID.NO.105
1453	PHSSSHWLRLPRIFC	SEQ.ID.NO.106
1454	HWLRLPRIFCSCPIG	SEQ.ID.NO.107
1455	PRIFCSCPIGENSPL	SEQ.ID.NO.108

TABLE 8

	DAY (mOD/min)			
Monkey #	0	57	68	96
1	3	5	2	2
2	4	6	12	10
3	7	6	10	8
4	7	6	8	8
5	5	9	20	15
6	11	8	10	12
7	11	23	51	30
8	7	30	70	22
9	1	7	5	3
10	2	6	6	4
11	3	7	14	8
12	6	9	15	6

5

Category	Item	Value	Unit	
Agriculture	Wheat	1200	kg	
	Rice	800	kg	
	Corn	500	kg	
	Soybeans	300	kg	
	Cotton	200	kg	
	Tea	100	kg	
	Oilseeds	400	kg	
	Fruits	600	kg	
	Vegetables	700	kg	
	Others	100	kg	
Livestock	Cattle	50	head	
	Pigs	30	head	
	Sheep	20	head	
	Goats	10	head	
	Poultry	100	head	
	Birds	50	head	
	Others	10	head	
	Fish	20	kg	
	Shellfish	10	kg	
	Others	5	kg	
Forestry	Timber	100	m ³	
	Wood	50	m ³	
	Other products	20	kg	
	Seeds	10	kg	
	Others	5	kg	
	Manufacturing	Textiles	50	kg
		Food processing	30	kg
		Chemicals	20	kg
		Metals	10	kg
		Others	5	kg
Services		Transport	10	kg
		Health	5	kg
		Education	5	kg
		Others	5	kg
		Total	Grain	2800
	Oilseeds		400	kg
	Fruits		600	kg
	Vegetables		700	kg
	Others		100	kg
	Livestock		110	head
Fish	20		kg	
Shellfish	10		kg	
Others	5		kg	